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Histidine Codons Appended to the Gene Encoding the RPO22 Subunit of Vaccinia Virus RNA Polymerase Facilitate the Isolation and Purification of Functional Enzyme and Associated Proteins from Virus-Infected Cells

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Vaccinia virus encodes a eukaryotic-like RNA polymerase composed of two large and six small subunit protein species. A replication-competent virus with 10 histidine codons added to the single endogenous J4R open reading frame was constructed. The altered migration of the 22-kDa subunit of RNA polymerase on SDS–polyacrylamide gel electrophoresis confirmed that J4R encoded the RPO22 subunit and that the mutant virus was genetically stable. The histidine-tagged RNA polymerase bound quantitatively to metal-affinity resins and was eluted in an active form upon addition of imidazole. Glycerol gradient sedimentation of the eluted fraction indicated that most of the RPO22 in infected cells is associated with RNA polymerase. Using stringent washing conditions, metal-affinity chromatography resulted in a several hundred-fold increase in RNA-polymerase-specific activity, and substantially pure enzyme was obtained with an additional conventional chromatography step. When mild conditions were used for washing the metal-affinity resin, the vaccinia virus-encoded capping enzyme, early transcription factor, and nucleoside triphosphate phosphohydrolase I specifically co-eluted with the tagged RNA polymerase, consistent with their physical association. The ability to selectively bind RNA polymerase to an affinity column provided a simple and rapid method of concentrating and purifying active enzyme and protein complexes.

INTRODUCTION

Poxviruses are large DNA viruses that replicate in the cytoplasm of infected cells and encode enzymes and factors for replication and transcription of their genomes (Moss, 1996). More than 20 highly conserved poxvirus genes are devoted to the biosynthesis of viral mRNA. Studies with vaccinia virus (VV) indicated that the multisubunit DNA-dependent RNA polymerase contains polypeptides encoded by at least eight genes: two large subunits of 147 and 132 kDa and six small subunits ranging from 35 to 7 kDa (Broyles and Moss, 1986; Patel and Pickup, 1989; Ahn *et al.,* 1990a,b, 1992; Broyles and Pennington, 1990; Quick and Broyles, 1990; Amegadzie *et al.,* 1991a,b, 1992). Of these, the two largest (RPO147 and RPO132) and the smallest (RPO7) have eukaryotic RNA polymerase subunit homologs. Additionally, RPO30 is related structurally to the mammalian transcription elongation factor TFIIS (Ahn *et al.,* 1990a). Another polypeptide, the RNA-polymerase-associated protein of 94 kDa (RAP94), is stably associated with RNA polymerase made at late times after infection and confers early promoter specificity (Ahn and Moss, 1992; Kane and Shuman, 1992; Ahn *et al.,* 1994; Deng and Shuman, 1994). Sedimentation studies suggested that additional proteins, including the VV early transcription factor (VETF), capping enzyme and a DNA-dependent ATPase (NPH I), are associated loosely with the RNA polymerase (Broyles and Moss, 1987). Genetic evidence suggests that the transcription system may be packaged as an even larger complex (Zhang *et al.,* 1994).

A simple and rapid method of isolating the VV RNA polymerase and associated proteins from infected cells would facilitate transcriptional and structural investigations. A polyhistidine tag has been used to purify recombinant proteins by immobilized metal-affinity chromatography (IMAC) under native or denaturing conditions (Hochuli *et al.,* 1988; Janknecht *et al.,* 1991; Van Dyke *et al.,* 1992). This procedure has been applied to *Escherichia coli* RNA polymerase (Kashlev *et al.,* 1993) but not to the more complex eukaryotic enzyme. By tagging an essential subunit of the VV RNA polymerase, we considered that the retention of viral replication competency would be a stringent test of the function of the modified enzyme. All of the subunits as well as RAP94 are probably essential as suggested by their conservation in a distantly related vertebrate poxvirus (Senkevich *et al.,* 1997), and mutations in some have been shown to confer a conditionally lethal temperature-sensitive (*ts*) phenotype (Seto *et al.,* 1987; Thompson *et al.,* 1989; Kane and Shuman, 1992). A strategy was devised to isolate a recombinant VV with a polyhistidine tag on the RNA polymerase by taking advantage of a mutant with a *ts* lesion that had been mapped to an RNA polymerase subunit. Here we

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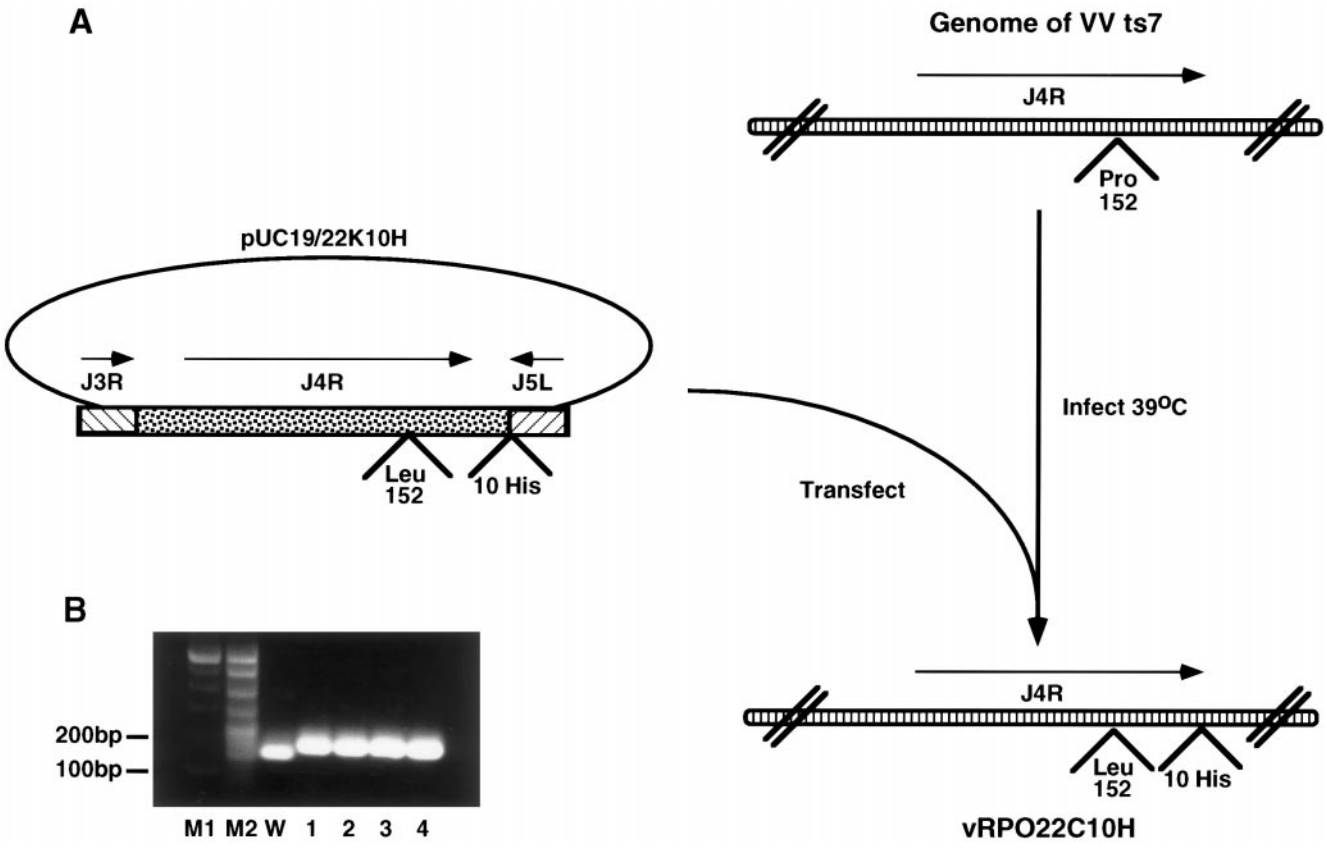


FIG. 1. Construction of a recombinant vaccinia virus encoding a histidine-tagged RNA polymerase subunit. (A) Procedure for isolating vRPO22C10H. The parental temperature-sensitive virus, VV *ts7*, has a proline substituted for leucine at amino acid 152. Cells were infected with VV *ts7* and transfected with pUC19/22K10H, which contained leucine at position 152 and 10 histidine codons at the C terminus. Viruses able to form plaques at 39°C were isolated. (B) Viral genomic DNA, derived from four independent plaque isolates, was analyzed by PCR using primers within and flanking the J4R gene. M1 and M2 are DNA markers; W, DNA from wild-type vaccinia virus; lanes 1–4, DNA from four viruses isolated as outline in (A).

demonstrate that the isolation strategy was efficient. The recombinant virus could be grown to high titer, and the RNA polymerase bound specifically to IMAC supports and provided a rapid method of isolating the enzyme and associated proteins from infected cells.

RESULTS

Isolation of a recombinant VV with a polyhistidine-tagged RNA polymerase subunit

We chose to add the polyhistidine tag to the RPO22 subunit of the VV RNA polymerase for several reasons. First, the molar ratio of RP022 to each large RNA polymerase subunit is three to four (Baroudy and Moss, 1980), so that the polymerase complex would have multiple polyhistidine tags to enhance binding to IMAC resins. Second, the C-terminal end of the J4R ORF encoding RPO22 is well separated from the neighboring J5L ORF so that addition of a polyhistidine tag was unlikely to interrupt coding or regulatory sequences. Third, conditional lethal mutants of VV with lesions in the J4R ORF were available (Thompson *et al.,* 1989), providing a method of selecting recombinant viruses by correction of the mutation. The mutant chosen, *ts*7, contains a Leu_{152} to Pro point mutation, which stringently prevents virus replication at 39°C. The strategy used to isolate a recombinant VV with a polyhistidine tag at the C terminus of the RPO22 subunit is depicted in Fig. 1A. We constructed transfer plasmids pUC19/22C6H and pUC19/22C10H, which contained a wild-type J4R ORF with 6 or 10 histidine codons added to the C terminus. The transfer plasmids were transfected into cells that had been infected with VV *ts*7. Because of the proximity of the *ts* lesion to the C terminus of the ORF, it seemed likely that many of the revertants selected by their ability to grow at the nonpermissive temperature would also have the polyhistidine tag, provided that the modification did not prevent RNA polymerase assembly or activity. The results were similar with the two plasmids so only data obtained with the 10-histidine construct are provided here. Of 14 initial plaques analyzed by polymerase chain reaction (PCR), 9 had the expected increase in the size of the J4R ORF due to the extra histidine codons. Four of these viruses were further plaque purified at 39°C and had a stable genotype (Fig. 1B). The recombinant VV, named vRPO22C10H, formed wild-type-size plaques at 39°C and could be

grown to high titer at 37°C, indicating that the polyhistidine tag had little or no adverse effect on virus replication. The replication competence of the mutant virus was corroborated by a one-step growth experiment, which showed the formation of infectious virus between 6 and 12 h after infection, and by [^{35}S]methionine pulse-labeling studies, which demonstrated the expected shift from early to late viral protein synthesis between 4 and 6 h after infection (data not shown).

Specific binding of tagged RNA polymerase to IMAC supports

Preliminary experiments indicated that the 10-histidine-tagged RNA polymerase from the cytoplasm of cells infected with vRPO22C10H bound to both cobalt resin (TALON, CLONTECH) and nickel agarose beads (Ni-NTA, Qiagen), eluting efficiently at 40 and 100 mM imidazole, respectively. RNA polymerase containing RPO22 with six histidines also bound to IMAC supports but eluted at lower imidazole concentrations. A typical cobalt IMAC experiment is illustrated in Fig. 2A. The loading material, unbound material, and column fractions were tested for RNA polymerase activity using a nonspecific single-stranded DNA template. Most of the RNA polymerase activity from cells infected with wild-type virus was detected in the flow through and wash fractions, indicating low affinity for the cobalt IMAC resin. In contrast, the majority of the active RNA polymerase from cells infected with vRPO22C10H bound and then eluted upon addition of 5–40 mM imidazole.

The samples were also analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting using antibodies to highly purified VV RNA polymerase (Jones and Moss, 1985) and to the RPO22 subunit (a gift of S. Shuman). In this experiment, the low-molecular-weight subunits were transferred most efficiently to the membrane and thus appeared more intense than the large ones. As anticipated, most of the viral RNA polymerase subunits from cells infected with wild-type VV were in the flow-through fraction, whereas most of the viral RNA polymerase subunits from cells infected with vRPO22C10H eluted with 40 mM imidazole (Fig. 2B). The SDS–PAGE analysis also revealed that the polyhistidine-tagged RPO22 subunit migrated slower than the wild-type polypeptide due to the additional amino acids (Fig. 2B). Moreover, there was no contamination with the faster-migrating polypeptide, indicating genetic stability of the mutant virus. In contrast to other subunits, RPO22 remained completely associated with the IMAC support after washing it with 6 M guanidinium hydrochloride or 8 M urea but eluted when imidazole was added, consistent with binding through its polyhistidine tag (data not shown).

Because both free RPO22 as well as RPO22 complexed with RNA polymerase would be expected to bind to the IMAC resin and the relative proportions of the two were unknown, the 40 mM imidazole fraction was analyzed by glycerol gradient sedimentation. The gradient fractions were subjected to SDS–PAGE and immunoblotting with RNA polymerase antisera. Most of the RPO22 sedimented in the fractions with other subunits (Fig. 2C) and RNA polymerase activity (not shown). A minor peak of RPO22 was detected near the top of the gradient, suggesting that some was present in smaller complexes or in a free form. A minor amount of RPO22 uncomplexed with RNA polymerase also was detected by anion exchange chromatography (data not shown). We concluded that the majority of the RPO22 polypeptide present at 20 h after infection is associated with RNA polymerase.

Purification of histidine-tagged RNA polymerase

The VV RNA polymerase usually is obtained by multiple chromatography steps from purified VV virions that have been disrupted with sodium deoxycholate and reducing agents (Baroudy and Moss, 1980; Spencer *et al.,* 1980). As shown above, the polyhistidine tag provides a means of directly isolating RNA polymerase from cytoplasmic extracts without detergents as well as from disrupted virions. The protocol used in the previous section was scaled up and modified. We started with 18 liters of HeLa cells infected with vRPO22C10H and passed the cell lysate through a column of Ni-NTA-agarose. To recover any unbound histidine-tagged RNA polymerase, the flow-through material was passed through a second column packed with fresh Ni-NTA-agarose. The columns were stringently washed with high salt, glycine, and 20 mM imidazole before eluting RNA polymerase with 100 mM imidazole. Considerable RNA polymerase activity was eluted with 100 mM imidazole from the first column (Fig. 3A), whereas insignificant activity was eluted with 100 mM imidazole from the second column (data not shown). The nature of the polymerase activity that did not bind Ni-NTA-agarose was not determined but probably included cellular RNA polymerases. The 100 mM imidazole fractions with highest RNA polymerase activity (fractions 2, 3 and 4; Fig. 3A) were applied to an HQ anion exchange column, which was eluted with a linear gradient of 0.4–0.8 M NaCl. The pooled RNA polymerase fractions were layered on to linear 15–35% glycerol gradients. The RNA polymerase activity sedimented as a sharp peak in fractions 9–12 (Fig. 3B), which coincided with the major polypeptides detected by SDS–PAGE and silver staining (Fig. 3C) and immunoblotting with antisera to RNA polymerase and RAP94 (not shown). The doublet of very slow migrating bands (>200,000 kDa) could include undissociated polymerase subunits that did not transfer well to membranes for Western blotting. Attempts to identify this material by

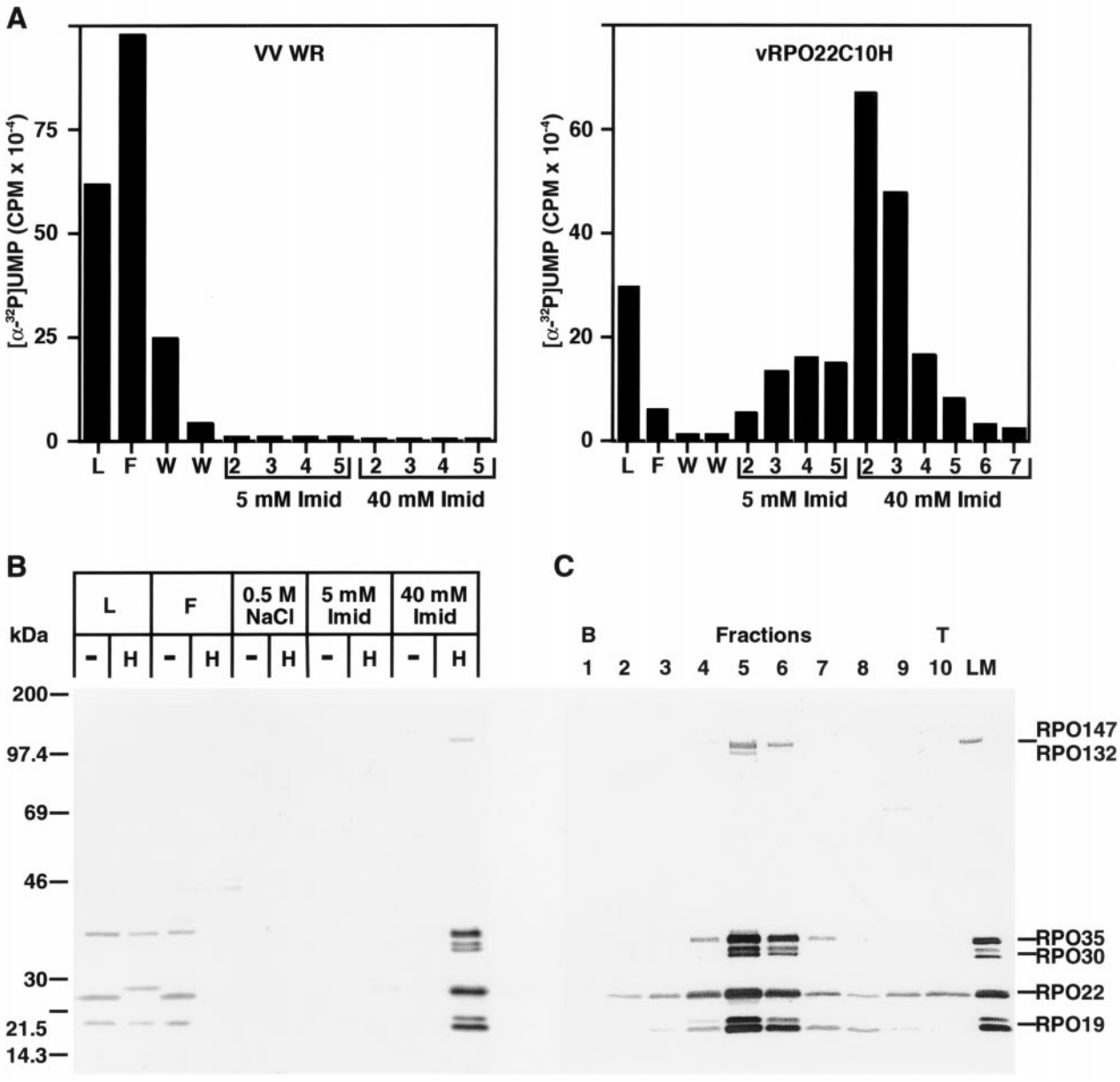


FIG. 2. Binding of histidine-tagged RNA polymerase to cobalt IMAC resin. (A) The cytoplasmic fraction of cells infected with wild-type strain VV WR or vRPO22C10H was incubated with 0.5 ml of TALON affinity resin, washed with 0.5 M NaCl, and eluted with imidazole. Fractions (0.5 ml) were collected after elution with 5 and 40 mM imidazole. RNA polymerase activity was determined. L, loading material; F, flow-through material; W, wash. (B) Western blot of fractions obtained in (A). The proteins resolved by SDS–PAGE were transferred to a membrane and incubated with antibody to RNA polymerase and RPO22. The masses in kDA of marker proteins are shown on the left. −, extracts from cells infected with wild-type VV; H, extracts from cells infected with vRPO2210H. (C) The pooled 40 mM imidazole fractions from cells infected with vRPO2210H was sedimented on a glycerol gradient and analyzed by Western blotting as in (B). The positions of RNA polymerase subunits are shown on the right. B, bottom of gradient; T, top of gradient.

mass spectroscopy revealed some peptides that were present in a 400-kDa cellular protein called giantin (unpublished data).

The polypeptide pattern of the histidine-tagged RNA polymerase purified from the cytoplasm was similar to that previously described for RNA polymerase from vaccinia virions including the presence of RAP94 (Ahn *et al.,* 1994) and multiple RPO30 (Ahn *et al.,* 1990a) and RPO19 (Ahn *et al.,* 1992) bands. SDS–PAGE analysis of the pooled fractions at each step of purification is shown in Fig. 4A. Calculations of the total RNA polymerase activity at each step of purification indicated a net increase, probably due to removal of inhibitors, and a 7000-fold purification. The greatest purification was achieved with the metal-affinity binding step and polymerase suitable for general purposes was obtained after HQ chromatography (Fig. 4B). The purified histidine-tagged RNA polymerase had more than two times the specific activity

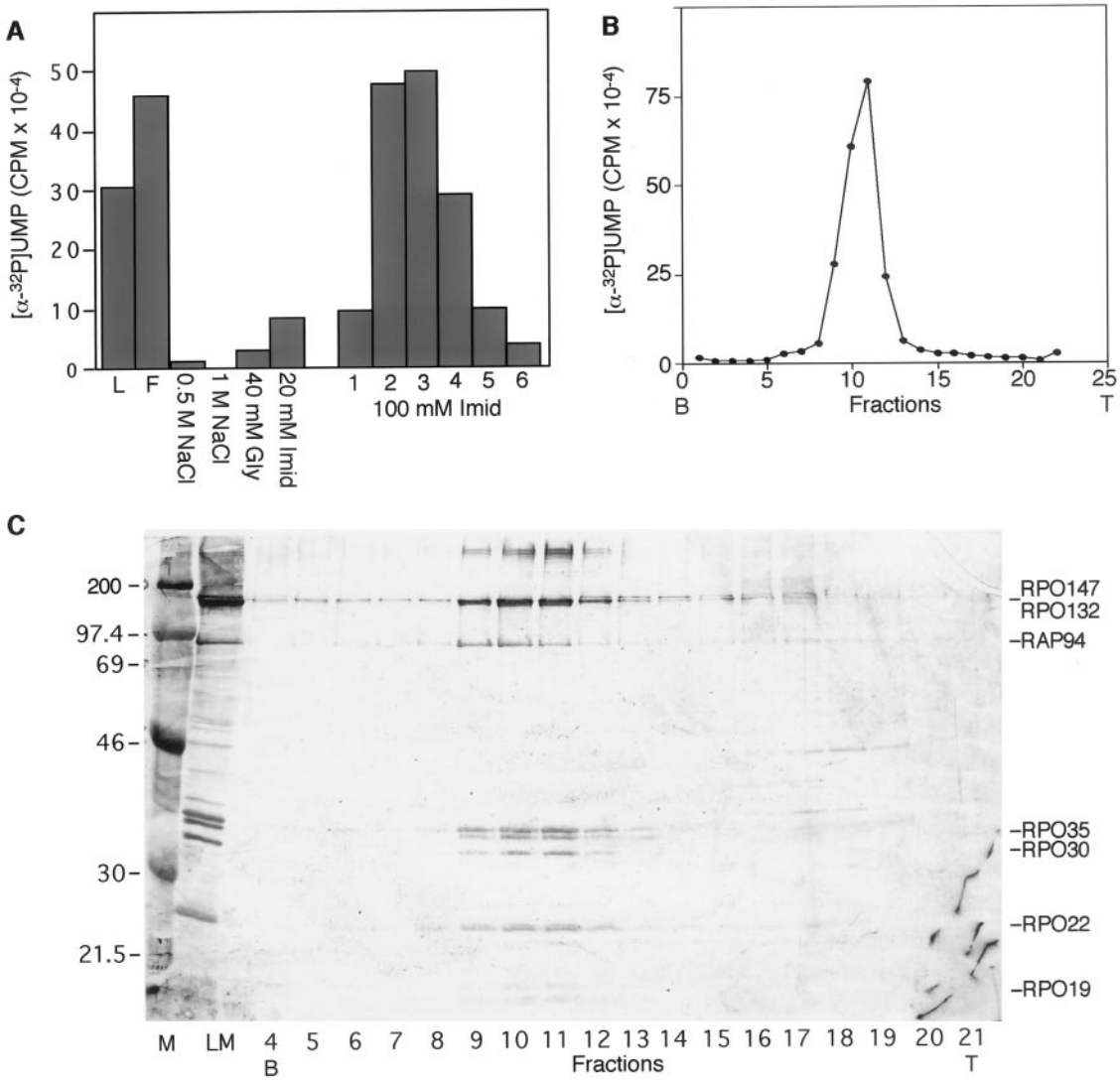


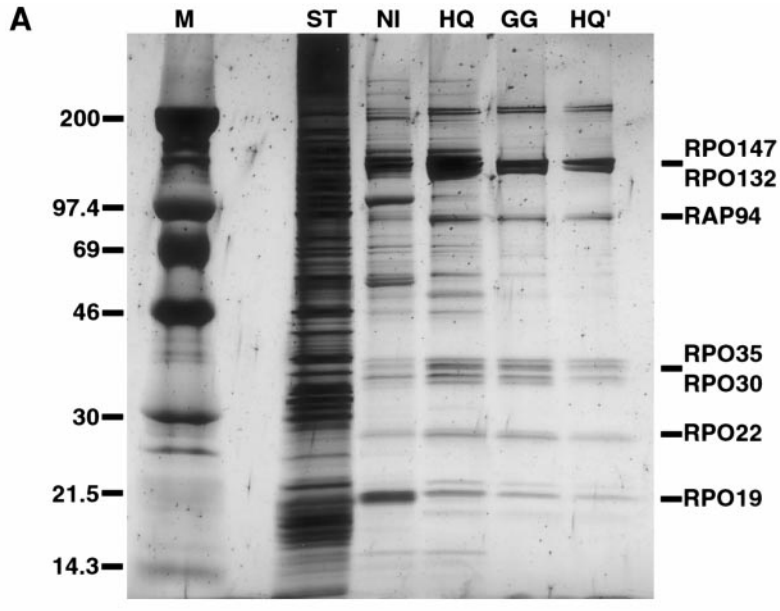
FIG. 3. Purification of histidine-tagged RNA polymerase. (A) Binding of histidine-tagged RNA polymerase to Ni-NTA beads. The beads were washed successively with 0.5 M NaCl, 1.0 M NaCl, 0.1 M NaCl plus 40 mM glycine (gly), 0.1 M NaCl plus 20 mM imidazole (imid), and 0.1 M NaCl plus 100 mM imidazole. The RNA polymerase activity in 10 μl aliquots was determined and then multiplied by the volumes of each fraction to give total activity. L, loading material; F, flow-through material. (B) RNA polymerase activity of glycerol gradient fractions. The pooled 100 mM imidazole fractions (A) were chromatographed on an HQ/M anion exchange column. Fractions with RNA polymerase activity were pooled and subjected to glycerol gradient centrifugation. RNA polymerase activity of gradient fractions are shown. B, bottom; T, top. (C). SDS–PAGE. Fractions from the glycerol gradient were subjected to SDS–PAGE and silver stained. LM, loading material. Masses in kDa of marker proteins (M) on left. Positions of RNA polymerase subunits on right.

previously reported for RNA polymerase purified from vaccinia virions (Baroudy and Moss, 1980) and 27 times the specific activity and twice the yield of that reported for RNA polymerase purified from vaccinia virus-infected cells (Nevins and Joklik, 1977).

Using a similar protocol, histidine-tagged RNA polymerase of comparable purity was isolated from cells infected with vaccinia virus in the presence of an inhibitor of DNA replication. The latter viral RNA polymerase, however, lacked RAP94, which is a late protein synthesized only after DNA replication (Ahn *et al.,* 1994).

Association of other viral proteins with metal-affinity purified RNA polymerase

By eliminating the high-salt and glycine wash steps, metal-affinity beads provided a way of isolating proteins that were weakly associated with the histidine-tagged RNA polymerase. Lysates of cells infected with wild-type VV or vRPO22C10H were incubated with Ni-NTA-agarose and then washed with 0.15 M NaCl and increasing concentrations of imidazole. Because of the mild washing conditions, the overall pattern of polypeptides that eluted with 100 mM imidazole was similar for both vi-



B

purification steps	volume (ml)	activity	total protein (μg)	specific activity
Starting	200	13807	20000	0.69
Nickel	30	39245	270	145
HQ	4.5	44232	20.25	2184
Glycerol	7	50239	10.5	4784

FIG. 4. Recovery and specific activity of histidine-tagged RNA polymerasy. (A) Silver-stained SDS polyacrylamide gel of fractions obtained during purification and concentration of histidine-tagged RNA polymerase. The amounts of total protein applied to each lane were as follows: starting material (ST) 2 μg; pooled fraction eluted from Ni-NTA (Ni) 180 ng; pooled fractions eluted from HQ/M anion exchange resin (HQ) 90 ng; and pooled fractions from glycerol gradient (GG) 30 ng. The lane marked HQ′ contained pooled glycerol gradient fractions that had been concentrated by step elution from an HQ/M column. Masses in kDa of markers are on the left. Positions of RNA polymerase subunits are on the right. (B) Recovery and specific activity after each step of purification.

ruses (Fig. 5A). On closer inspection of the silver-stained gel, however, the viral RNA polymerase bands were detected specifically in the 100 mM imidazole fraction from vRPO22C10H-infected cells, indicating a considerable enrichment. Moreover, RNA polymerase bands were undetectable by immunoblotting of the 100 mM imidazole fraction of cells infected with wild-type virus but were concentrated in the same fractions from cells infected with vRPO22C10H (Fig. 5B). Therefore the concentration of additional polypeptides in the 100 mM imidazole fraction from cells infected with vRPO22C10H (but not in the equivalent fraction from cells infected with wild-type virus) would indicate that they are associated with the histidine-tagged RNA polymerase. Western blotting demonstrated the concentration of vaccinia virus early transcription factor (VETF) encoded by the D6R and A7L ORFs, the capping enzyme encoded by the D1R and D12L ORFs, and nucleoside triphosphate phosphohydrolase (NPH) I encoded by the D11L ORF in the 100 mM imidazole fractions of vRPO22C10H (Fig. 6). In contrast, Western blotting indicated that there was no specific association of the polyhistidine-tagged RNA polymerase with the late transcription factors encoded by the G8R, A1L, A2L, and H5R ORFs or the two poly(A) polymerase subunits encoded by the E1L and J3R ORFs or the DNA topoisomerase encoded by the H6R ORF under the same conditions (data not shown). Antibodies to several other viral proteins were also tested but the Western blots were not adequate to draw conclusions regarding association with RNA polymerase. As expected from the Western blotting, the fractions containing RNA polymerase and VETF were able to transcribe a DNA template with an early promoter but not a late promoter (data not shown). We also demonstrated the transcription of a DNA template containing an intermediate promoter using Ni-NTA-agarose-purified RNA polymerase from cells infected with VV in the presence of an inhibitor of DNA replication (data not shown).

DISCUSSION

The subunits of the vaccinia virus RNA polymerase have not been extensively characterized by biochemical

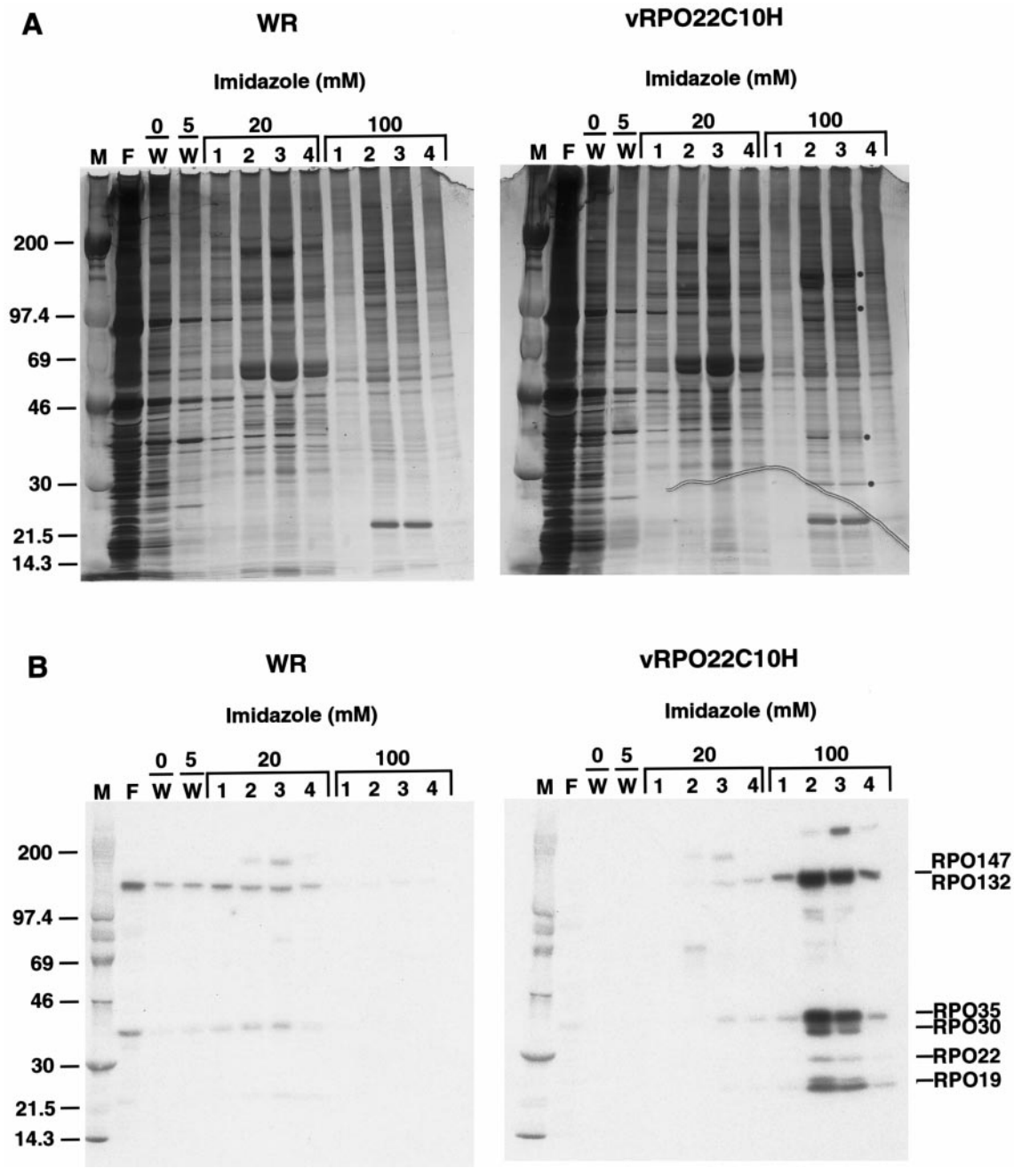


FIG. 5. IMAC chromatography using mild washing conditions. (A) Extracts of cells infected with VV WR or vRPO22C10H were applied to Ni-NTA-affinity columns. The beads were washed successively with buffers containing 0.15 M NaCl and 0, 5, 20 and 100 mM imidazole in 0.15 M NaCl. At the higher imidazole concentrations, 0.5-ml fractions numbered 1–4 were collected. Samples were subjected to SDS–PAGE and silver staining. Putative RNA polymerase subunits are marked by dots. Masses in kDa of marker proteins are shown on the left. M, marker proteins; F, flow-through material, W, washes. (B) Western blotting. The samples in (A) were also analyzed by Western blotting using antibody to RNA polymerase. Abbreviations as (A). Masses in kDa are shown on left and RNA polymerase subunits on the right.

or genetic methods. The location of the gene encoding RPO22 was originally determined using antibody raised against purified RNA polymerase to immunoprecipitate a 23-kDa *in vitro* translation product of early mRNA that had been hybridized to the *Hin*dIII J fragment of the VV genome (Jones *et al.,* 1987). DNA sequencing revealed an ORF (now called J4R) within the *Hin*dIII J fragment that was capable of encoding a 22-kDa polypeptide that was inferred to be RPO22 (Broyles and Moss, 1986). Although the J4R ORF had no homology with subunits of other RNA polymerases, the identification was consistent with the defective late gene expression phenotype of two mutants with *ts* lesions that mapped to this ORF (Hooda-Dhingra *et al.,* 1989; Thompson *et al.,* 1989). The present demonstration, that histidine codons added to the J4R ORF decrease the electrophoretic mobility of RPO22 and

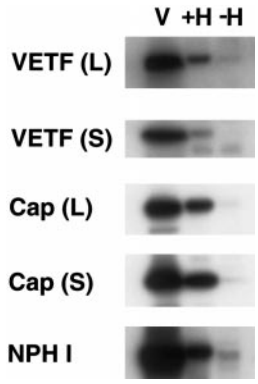


FIG. 6. RNA polymerase-associated proteins. Fractions 2 and 3 from the 100 mM imidazole elution in Fig. 5 were analyzed by Western blotting using antibodies to the large (L) and small (S) subunits of VETF and capping enzyme and NPH I. Dissociated proteins from purified VV were analyzed in parallel to provide markers for enzymes and factors. V, purified VV virions; +H, from cells infected with vRPO22C10H; −H, from cells infected with VV WR.

cause the RNA polymerase to bind tightly to metal-affinity beads, provides direct evidence that J4R encodes an integral subunit of the vaccinia virus RNA polymerase. At late times after infection, only relatively small amounts of RPO22 were uncomplexed with RNA polymerase.

The procedure used to make the histidine-tagged RNA polymerase may have general applicability to other viral proteins. By modifying the existing J4R ORF within the vaccinia virus genome, rather than inserting an additional recombinant copy, we could be assured that RPO22 was expressed at the correct time and level, the histidine-tagged RNA polymerase was biologically active, and all copies of the subunit were tagged. An efficient selection protocol was based on starting with a mutant vaccinia virus containing a *ts* lesion in the J4R ORF and simultaneously correcting the defect and adding histidine codons. The majority of mutants that formed plaques at the elevated temperature had the polyhistidine tail. A similar procedure might be used to tag other RNA polymerase subunits or RAP94 for which *ts* lesions have been mapped (Seto *et al.,* 1987; Thompson *et al.,* 1989; Hooda-Dhingra *et al.,* 1990; Kane and Shuman, 1992). By tagging RAP94, RNA polymerase specific for early transcription might be obtained.

We chose to add the polyhistidine tag to RPO22 in part because there was evidence for three to four copies per RNA polymerase molecule (Baroudy and Moss, 1980). We hoped that this stoichiometry would ensure a high avidity for metal-affinity beads. In this respect, the histidine-tagged RNA polymerase bound quantitatively to IMAC supports. We found, however, that the 10-histidine-tagged RNA polymerase eluted from cobalt and Ni-NTA at relatively low concentrations of imidazole, 40 and 100 mM, respectively. This could be due to the large surface of the multisubunit RNA polymerase or to poor exposure of the C terminus of RPO22.

The polyhistidine tag provided an extremely useful first step in the purification of RNA polymerase from cytoplasmic extracts of infected cells. The polymerase remained bound to IMAC beads in the presence of high concentrations of salt and glycine, which removed many other proteins, and provided a several hundred-fold purification. The polymerase was purified an additional 10-fold by HQ chromatography. At this stage, the major bands were polymerase subunits as determined by SDS–PAGE, and most of the contaminating polypeptides were removed by glycerol gradient centrifugation. The specific activity of the RNA polymerase was higher than that previously reported for the enzyme purified by conventional methods from either virus particles (Baroudy and Moss, 1980) or infected cells (Nevins and Joklik, 1977).

The polyhistidine tag was also used to isolate and identify proteins weakly associated with the VV RNA polymerase. In a prior study (Broyles and Moss, 1987), transcriptionally active RNA polymerase was found to cosediment with capping enzyme and NPH I. To demonstrate that these components were part of a single complex and not multiple cosedimenting species, we applied extracts from cells infected with wild-type vaccinia virus or vRPO22C10H to separate but identical Ni-NTA beads. The columns were washed to remove untagged RNA polymerase and then with 100 mM imidazole to recover the histidine-tagged enzyme and any associated proteins. The 100 mM wash, containing proteins from cells infected with wild-type vaccinia virus, served as a control for polypeptides that may have remained bound to the IMAC resin in the absence of RNA polymerase. Significantly, VETF, capping enzyme and NPH I were specifically associated with the histidine-tagged RNA polymerase fraction. However, this association was disrupted by more stringent IMAC conditions or further purification steps (Katsafanas and Moss, unpublished). Nevertheless, this complex appears to have functional significance because VETF is required for binding of the RNA polymerase to the promoter and transcription initiation (Broyles *et al.,* 1988; Li and Broyles, 1993; Baldick *et al.,* 1994), whereas capping enzyme and NPH I mediate transcription termination (Shuman *et al.,* 1987; Deng and Shuman, 1998). Although genetic studies suggested that poly(A) polymerase and topoisomerase were complexed with RNA polymerase during virion assembly (Zhang *et al.,* 1994), these enzymes eluted from the IMAC column before that of the histidine-tagged RNA polymerase, indicating that any association must be quite weak.

In summary, the engineering of a replication competent VV with a polyhistidine tag at the end of the J4R ORF has allowed us to confirm that this ORF encodes RPO22 and that RPO22 is an integral subunit of VV RNA polymerase. The tag provided a rapid procedure for concentrating and purifying RNA polymerase and associated proteins from extracts of VV-infected cells. Experience with a polyhistidine-tagged *E. coli* RNA polymerase

(Kashlev *et al.,* 1993; Tang *et al.,* 1996), suggests that the present construct is likely to be useful for studies of VV RNA polymerase assembly and for a mechanistic analysis of transcription.

MATERIALS AND METHODS

Cells and virus

HeLa S-3 suspension cells (ATCC CCL2.2) and monolayer cultures of BS-C-1 cells (ATCC CCL26 were propagated as described (Earl *et al.,* 1997). Stocks of VV strain WR (ATCC VR 1354) and vRPO22C10H were prepared in HeLa cell monolayers at 37°C as described (Earl *et al.,* 1997). VV *ts*7 (Thompson *et al.,* 1989) was propagated in a similar manner except that the temperature was 31°C.

Recombinant DNA

Plasmid pUC13 containing the VV strain WR *Hin*dIII-J fragment was cleaved with *Hin*cII and *Kpn*I, and the resulting fragment of 1.4 kb was purified by gel electrophoresis and ligated to *Hin*cII and *Kpn*I cleaved pUC19 (GenBank Accession No. X02514). The new plasmid was named pUC19/22k. To introduce a 10-histidine tag at the C terminus of the J4R open reading frame (ORF), two sets of primers were designed: 5′GAGAACATGAGACTGACTCGAGTTACC and 5′ttagctagaatggtgatgatggtgatgatggtgatgatgaccATCAGCGACTGAAATAACAGATCTATCGGC; and 5′ggtcatcatcaccatcatcaccatcatcaccattctagctaaTCAATTAGTAGAGATGAGATAAGAACATTAT and 5′GCCCCATCCATAGCACCTAGTCTACCATC. The upper and lower case letters refer to VV and non-VV sequences, respectively. Each set of primers was used separately for PCR with VV strain WR DNA as the template. The resultant products were 864 and 750 bp, respectively. A portion of each reaction was combined and allowed to undergo 10 cycles of amplification, at which time oligonucleotides 5′GAGAACATGAGACTGACTCGAGTTACC and 5′GCCCCATCCATAGCACCTAGTCTACCATC were added, followed by 25 more cycles of amplification. The reaction yielded a 1575-bp fragment that was purified by gel electrophoresis and digested with *Bbs*I and *Bst*XI, giving rise to a 440-bp fragment internal to these cleavage sites. This product was gel purified and ligated with pUC19/22k, which had been digested with the same enzymes and purified away from the 401-bp product internal to these enzyme sites. The new plasmid was named pUC19/22k10H.

Recombinant virus construction

Recombinant viruses were constructed as described (Earl *et al.,* 1997) with modifications as indicated. Six-well dishes (5×10^5 cells/well) were infected with VV *ts*7 virus at a m.o.i. of 0.03 plaque forming units (PFU)/cell. After 4 h at 31°C, the infected cells were transfected with pUC19/22k10H mixed with lipofectin (GIBCO), and all subsequent incubations were carried out at 39°C. After another 4 h, the liquid medium was replaced with medium in 1% low-melting-point agarose. At 24 h, a second overlay of 1% low-melting-point agarose in medium with 100 μg/ml of neutral red was applied. After 48 h, the plaques were aspirated with a pipette and mixed with 0.2 ml of 1 mM Tris–HCl pH 9.0. The suspension was frozen and thawed three times, and 10 μl was used as a template with PCR primers that amplified the C-terminal coding sequence of the J4R ORF essentially as described (Zhang and Moss, 1991). The PCR products were analyzed by gel electrophoresis. A recombinant VV with a 10-histidine tag at the C terminus of the gene encoding RPO22 was isolated by additional plaque purification steps and named vRPO22C10H.

Binding of RNA polymerase to cobalt IMAC resin

Suspended HeLa S3 cells (5×10^8) were infected with 5 PFU/cell of VV WR or vRPO22C10H, incubated for 24 h at 37°C, collected by centrifugation, washed with phosphate buffered saline (PBS), and resuspended in 4.5 ml of buffer A [10 mM Tris–HCl (pH 8.0), 1 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF)] with 10 mM NaCl and 5 mM $MgCl_2$. Subsequent steps were carried out at 0 to 4°C. After 5 min on ice, the cells were Dounce homogenized and the lysate was clarified by successive centrifugations at 1000 *g* for 10 min and 21,500 *g* for 15 min. The high-speed supernatant was mixed with an equal volume of buffer B [buffer A plus 0.19 M NaCl, 1 mM imidazole, 0.02% (v/v) Triton X-100, 20% (v/v) glycerol)] and incubated with 0.5 ml of cobalt IMAC resin (TALON, CLONETECH) overnight with mixing. The resin was then poured into a column and washed with: 10 vol of buffer C (buffer A plus 0.01% Triton X-100 and 10% glycerol) with 0.1 M NaCl and 0.5 mM imidazole; 10 vol of buffer C with 0.5 M NaCl and 0.5 mM imidazole; 10 vol of buffer C with 5 mM imidazole and 0.1 M NaCl; and 5 vol of buffer B with 40 mM imidazole and 0.1 M NaCl.

Purification of RNA polymerase

Approximately 9×10^9 HeLa cells were infected with vRPO22C10H, collected, and lysed essentially as described above except that the lysis buffer also contained 10 mM NaF. After 5 min swelling, the cells were Dounce homogenized, and the lysate was mixed with 1 vol of buffer A supplemented with 0.83 M NaCl, 1 mM imidazole, 34% glycerol, and 0.4% (v/v) Tween 20. After 1 h, the suspension was centrifuged at 10,000 *g* for 15 min, and the supernatant was incubated with 25 ml of nickel IMAC agarose beads (Ni-NTA, Qiagen) overnight with mixing. The beads were poured into a column and washed successively with 5 vol of buffer D (buffer A plus 0.5 mM

imidazole, 10 mM NaF, 17% glycerol) with: 0.5 M NaCl; 1 M NaCl; 0.1 M NaCl, 40 mM glycine; 0.1 M NaCl, 20 mM imidazole; 0.1 M NaCl, 100 mM imidazole. The pooled RNA polymerase from the 100 mM imidazole fractions (30 ml) was applied to a 4.6 × 100 mm HQ/M anion exchange column (Perseptive Biosystems), which was washed with 5 ml of buffer E [10 mM Tris–HCl (pH 8), 0.1 mM EDTA, 1 mM DTT, 0.01% Triton X-100, 10% glycerol) containing 0.1 M NaCl and developed with a 10 ml linear gradient from 0.4 to 0.8 M NaCl in buffer E at a flow rate of 0.5 ml/min using a Waters 650 E purification system. RNA polymerase peak fractions were pooled, and 0.75 ml was layered onto each of six 11-ml 15–35% (vol/vol) glycerol gradients in 0.5 M NaCl, 10 mM Tris–HCl (pH 8), 0.1 mM EDTA, 1 mM DTT, and 0.01% (v/v) Triton X-100. The gradients were placed in a SW41 rotor and centrifuged in a Beckman L8–80M ultracentrifuge for 26 h at 41,000 rpm.

Isolation of RNA polymerase with associated proteins

HeLa suspension cells (1.5×10^8) were infected with 5 PFU/cell of VV strain WR or vRPO22C10H. After 20 h, the cells were collected by centrifugation, washed with PBS and were lysed as described above for purification of RNA polymerase. The lysate was centrifuged at 1000 *g* for 10 min and the supernatant centrifuged at 21,500 *g* for 15 min. The high-speed supernatant was mixed with an equal volume of 0.29 M NaCl, 10 mM Tris–HCl (pH 8), 1 mM β-mercaptoethanol, 1 mM imidazole, 30% glycerol and then incubated with 1.5 ml of Ni-NTA agarose beads overnight with mixing. The beads were placed in a column and washed with 10 column volumes of 0.15 M NaCl, 10 mM Tris–HCl (pH 8), 1 mM β-mercaptoethanol, 0.5 mM imidazole, 15% glycerol. The column was then washed with 10 vol of buffer F [0.15 M NaCl, 10 mM Tris–HCl (pH 8), 1 mM β-mercaptoethanol, 0.01% Triton X-100, 15% glycerol] containing 5 mM imidazole, followed by 10 vol of buffer F with 20 mM imidazole, and then 5 vol of buffer F with 100 mM imidazole.

RNA polymerase activity

VV RNA polymerase was assayed as described (Baroudy and Moss, 1980) in 0.1 ml of 50 mM Tris–HCl (pH 8), 2 mM DTT, 1 mM ATP, GTP, CTP; 0.01 mM UTP; 5 μCi [α-^{32}P]UTP (3 μCi/nmol); 4 mM $MnCl_2$; 1 μg of heat denatured calf thymus DNA; and 10 μl of the protein fraction to be assayed. Incubations were carried out at 37°C for 30 min and stopped by pipetting the reaction mixture onto Whatman DE81 ion exchange paper and washing three times in 0.5 M Na_2HPO_4, twice in H_2O, and once in 100% ethanol. After drying, the radioactivity was measured by scintillation spectrophotometry. Activity was expressed as counts per min or as units (pmol UMP incorporated in 30 min at 37°C/μg of protein).

SDS–PAGE

Proteins were resolved on a 4–20% gradient or 10% polyacrylamide SDS gel and visualized using Silver Stain Plus Kit (Bio-Rad Laboratories). For immunoblotting, proteins were transferred to a nitrocellulose membrane (Micron Separations) and probed with antibodies. Then the membranes were either incubated with ^{125}I-labeled protein A and autoradiographs prepared or incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) and developed using the Phosphatase Substrate System (Kirkegaard and Perry Laboratories). The sources of rabbit polyclonal antibodies were as follows: viral RNA polymerase (Jones *et al.,* 1987), RAP94 (Ahn and Moss, 1992), VETF (gift of C. Cassetti); nucleotide phosphohydrolase I (NPHI) (Paoletti *et al.,* 1974), RPO147, RPO132, and RPO22 (gifts of S. Shuman), capping enzyme subunits (gift of N. Harris).

Protein determination

Samples (10 μl) were assayed using the Bio-Rad Protein Assay following the manufacturer's specifications.

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